ZOOLOGICAL SCIENCE 7: 1–16 (1990)

REVIEW

Molecular Assembly and Evolution of Multi-Subunit Extracellular Annelid Hemoglobins

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ABSTRACT—An extracellular annelid hemoglobin is a multi-subunit protein with a molecular weight of $3–4\times10^6$ and exhibits a hexagonal bilayer of twelve submultiples. Recent advances in studies on the molecular evolution and assembly of the huge annelid hemoglobins are summarized. Sequence determinations of the eight polypeptide chains of the multi-subunit hemoglobins of the polychaete *Tylorrhynchus heterochaetus* and the oligochaete *Lumbricus terrestris* have provided fundamental information on the common molecular architecture and the phylogeny of these huge dioxygen-carrying proteins. In addition, morphological studies using scanning transmission electron microscopy and conventional transmission electron microscopy with image analysis have revealed the tetrahedeal structure of the submultiple. A new nomenclature '*a*', '*A*', '*b*', and '*B*', is proposed for the four basic constituent chains common to oligochaete and polychaete hemoglobins based on their homology. Phylogenetically, these heme-containing chains can be separated into two strains '*A*', and '*B*'. According to the symmetrical '192-chain' model, the multi-subunit hemoglobins might be represented as '$(aAbB)_{48}$'. The minimum entity '*aAbB*' that consists of a monomeric chain '*a*' and a disulfide-bonded trimer '*AbB*' may correspond to one unit in the tetrahedral of the submultiple in electron microscopic appearance. On the basis of recent information, earlier models are evaluated as well as the 'bracelet' model, in which the minor subunits D1 and D2 without heme have a key role in linking the complexes of subunit *a* and subunit *AbB* together.

INTRODUCTION

Ever since Svedberg [1–3] found that an extracellular annelid hemoglobin is a huge protein and consists of either about 144 or 192 polypeptide chains, an outstanding problem has been to construct a common model for the molecular architecture of annelid hemoglobins.

Ten years ago, we decided to determine the molecular weight of each constituent chain of hemoglobin of the polychaete *Tylorrhynchus heterochaetus* by analyzing its amino acid sequence [4, 5]. Our results have provided much information on the molecular architecture and the evolution of giant annelid hemoglobins [6–11]. Similar studies on hemoglobin of the oligochaete *Lumbricus terrestris* have progressed concurrently in other laboratories [12–14]. Four major species of constituent chains with heme have been isolated from each of these polychaete and oligochaete hemoglobins and have been sequenced. Moreover, Vinogradov and his colleagues recently reported that non-heme chains with molecular masses of 31–37 kDa act as a scaffold for the complexes of two types of heme-containing subunits 'monomers' and disulfide-bonded 'trimers' [15–17]. Morphological studies by scanning transmission electron microscopy ([1]STEM) [18–20] and conventional transmission electron microscopy (CTEM) with image

Received October 16, 1989

[1] Abbreviations used: STEM, scanning transmission electron microscopy; CTEM, conventional transmission electron microscopy; SDS-PAGE, sodium dodecyl sulfate-polyyacrylamide gel electrophoresis; SAXS, small-angle X-ray scattering.

analysis technique [21, 22] have also provided information for constructing of a common model of giant hemoglobins.

These recent findings are all consistent with a common model for the molecular architecture of multi-subunit annelid hemoglobins consisting of about 200 polypeptides including four species of 192 heme-containing chains and some non-heme chains, although the detailed subunit assembly is still controversial. In particular, it is still uncertain whether annelid hemoglobins contain common non-heme chains with molecular masses of about double those of heme-containing chains. The 288 disulfide bonds present in the heme-containing chains of a molecule must be important for interloking the whole complex [11, 14] and perhaps the non-heme chains may link the complexes of heme-containing subunits together [16, 23]. With regard to molecular phylogeny, two globin strains have been found in both polychaete and oligochaete hemoglobins [9, 23, 24]. In this article we introduce a new nomenclature for the four main heme-containing chains common to oligochaete and polychaete hemoglobins so that future 'monomeric' and 'trimeric' globin chains can be readily compared. Although non-heme chains are expected to be 'linkers' that consist of two domains of globin and conserve the drastic evolutionary history of the hemoglobins, these chains must be examined further, especially in terms of amino acid or DNA sequences, to be identified as real components. Since the major constituent chains with heme have all been found to be homologous with a vertebrate myoglobin, the old name for giant annelid hemoglobins, 'erythrocruorin', is no longer appropriate.

HISTORICAL BACKGROUND

Sixty years ago, Svedberg [1] developed a method for determining the molecular weight of a protein by centrifugation, and using this method he found a variety of globins ranging in molecular weight from 17,000 for myoglobin to about 3 million for annelid extracellular hemoglobins. As the annelid extracellular hemoglobin is a huge protein, Svedberg and Eriksson [2] thought that its protein portion have completely different chemical properties from those of vertebrate hemoglobins, and they revived the name "erythrocruorin", originally used for the red blood pigment of the invertebrates by Ray Lankester in 1868 [25]. No satisfactory explanation of the evolutionary relationships between different sizes and forms of globins was proposed for many years. But in 1960, in X-ray diffraction studies Perutz [26] observed similarity in the three dimensional structures of vertebrate myoglobin and hemoglobin. Now, more than 250 globin chains have been sequenced [27] and a phylogenetic tree for their molecular evolution has been constructed [28]. Thus the homologies of diverse hemoglobins have been established and these molecules have been shown to be members of the globin family.

Kimura [29] proposed the neutral theory for molecular evolution based largely on the primary structures of vertebrate globin chains. Recently, however, attention has been directed into the structural diversity of invertebrate hemoglobins, with the expectation of finding some drastic mutations besides the point mutations that have already been analyzed in vertebrate globin chains. Based on the quarternary structures, Vinogradov [30] has classified hemoglobins into four types: (*a*) Single-domain, single-subunit molecules consisting of a single polypeptide chain of about 16 kDa containing one heme group; (*b*) two domain, multi-subunit hemoglobins, ranging in size from 250 to 800 kDa and consisting of 30–40 kDa chains, each containing two heme-binding domains; (*c*) multi-domain, multi-subunit hemoglobins, consisting of two or more long polypeptide chains each containing 8–20 heme-binding domains connected lineally; (*d*) Single-domain, multi-subunit hemoglobins, consisting of aggregates of several small subunits, some of which are disulfide-bonded and not all of which contain heme. The giant extracellular annelid hemoglobins are of type *d*. Oligochaetes have only this type of hemoglobin. On the other hand, polychaetes have various types of pigments such as monomeric and polymeric intracellular hemoglobins, monomeric extracellular hemoglobins, and multi-subunit extracellular hemoglobins and chlorocruorins. In some cases, two types of hemoglobins are present in a single species [31–33]. Chlorocruorins are green, but they are considered to be

homologous with multi-subunit hemoglobins because of their similarities to the latter in size and shape [3, 34]. Annelid multi-subunit hemoglobins and chlorocruorins can also be characterized by the appearance of a double-layered hexagonal array of twelve submultiples [34–36]. These hemoproteins appear to consist of 'monomers', 'dimers', disulfide-bonded 'trimers' and disulfide-bonded 'tetramers' in various proportions and combinations depending upon the species [30]. The molecular masses of unit chains in all these forms except 'dimers' are comparable to those of vertebrate myoglobins [4, 12, 30]. Most preparations of these hemoglobins and chlorocruorins exhibit the presence of some 'dimer' components with molecular masses of 31–37 kDa, but they have not yet been well characterized. The relationships between the oxygen-dissociation curve and the structure of the annelid hemoglobins has been studied extensively [37–46]. The present review is, however, limited mainly to recent progress in studies on the molecular evolution and assembly of the multi-subunit extracellular annelid hemoglobins.

Many workers have studied different hemoglobins and have proposed models for their molecular assembly based on their results obtained by various methods [for earlier reviews, see Refs. 47–52]. For analysis of the whole molecule, precise measurements of basic parameters, such as the molecular weight of the whole molecule and the constituent chains, and the iron or heme content are necessary. The values reported for the molecular masses of whole molecules vary from about 3,000 kDa to 4,000 kDa [3, 40, 53]. The molecular masses of major constituent chains estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) range from 11 kDa to 19 kDa [54–56], whereas the molecular masses of the constituent chains estimated by centrifugation are 22–23 kDa [57, 58]. The minimum molecular mass per heme observed also ranges from 17 kDa to 28 kDa [54, 59–61]. Thus, even very recently it was difficult to justify the reported values of these parameters and to assign the heme group to the disulfide-bonded 'trimer' [15]. As pointed out by many workers [62], it is also difficult to determine the exact number of chains per whole molecule, because in estimating the molecular weight of the whole molecule any error in the minimum molecular weight is multiplied by about '144' or '192'.

MOLECULAR SHAPE

Figure 1 shows typical STEM images of polychaete and oligochaete hemoglobins, which consist of double layered hexagonal submultiples [11]. In the central cavity of the molecule, there are faint indications of protein masses that appear to be anchored to the surface of all 12 submultiples. In the view of *Tylorrhynchus* hemoglobin from the top (Fig. 1A), each submultiple appears to be composed of three globular units with a tiny hole at the center. The side view of a submultiple (Fig. 1B) also shows three identical units. From these findings the simplest steric model of a submultiple is a tetrahedral structure, the fourth unit being masked by the others both in the top and side views. The size of *Tylorrhynchus* hemoglobin is 28.4 nm in vertex-to-vertex diameter and 18.2 nm in height, as listed in Table 1 with the values for *Lumbricus* hemoglobin for comparison [19]. The high resolution STEM method was developed by Crewe of Chicago University [63] and first used to examine *Lumbricus* hemoglobin by Kapp and Crewe in the collaboration with Vinogradov of Wayne State University [18]. Recently, Vinogradov and his collaborators [15–17, 20] suggested a role of the filament structure in the central cavity of the hemoglobin molecule as a scaffolding or linker for the submultiples. By two dimensional image analysis and reconstruction by optical and computed methods of electron micrographs, Ghiretti Magaldi and her colleagues [21, 22, 64] demonstrated the tetrahedral structure of the each submultiple of an extracellular hemoglobin and a chlorocruorin as shown is Figure 2. These electron microscopic studies indicated the existence of 48 tetrahedral units in the whole molecule. However, the materials found by STEM in the central cavity could not be observed by CTEM with image analysis [22].

Several multi-subunit annelid hemoglobins have been examined by small-angle X-ray scattering (SAXS) [65–70], which is a useful method for obtaining information on the quarternary structure

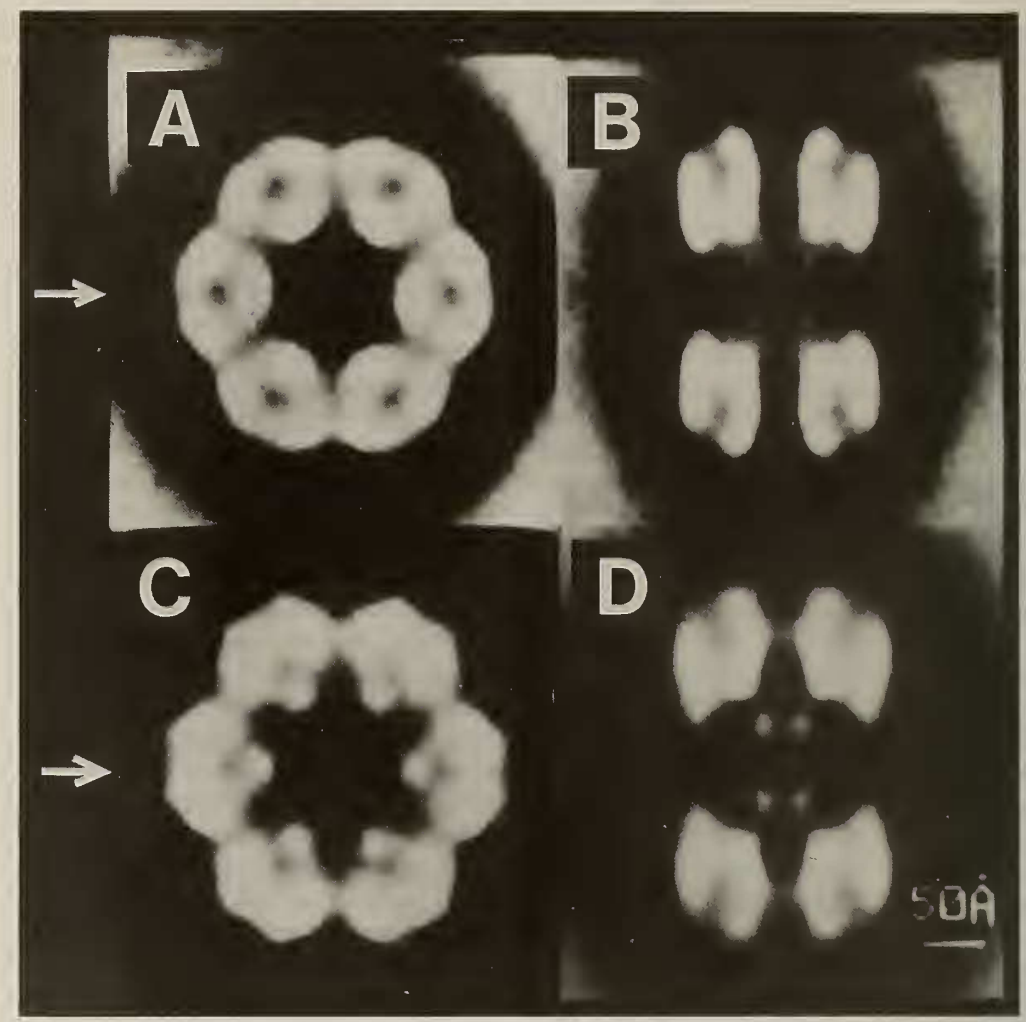


FIG. 1. STEM images of *Tylorrhynchus* (A and B) and *Lumbricus* (C and D) hemoglobins. A and C, and B and D are top and side views, respectively. Note the faint protein mass in the central cavity. (from Suzuki, T., Kapp, O. H. and Gotoh, T. [11]. Reprinted with permission from the American Society for Biochemistry & Molecular Biology.)

TABLE 1. Molecular dimensions of *Tylorrhynchus* and *Lumbricus* hemoglobins determined by STEM

Hemoglobin	Vertex-to-vertex diameter (nm)	Height (nm)	Central hole (nm)	Ref.
Tylorrhynchus	28.4	18.2	8.8	[11]
Lumbricus	30.7	20.1	8.8	[19]

(from Suzuki, T., Kapp, O. H. and Gotoh, T. [11]. Reprinted with permission from the American Society for Biochemistry and Molecular Biology.)

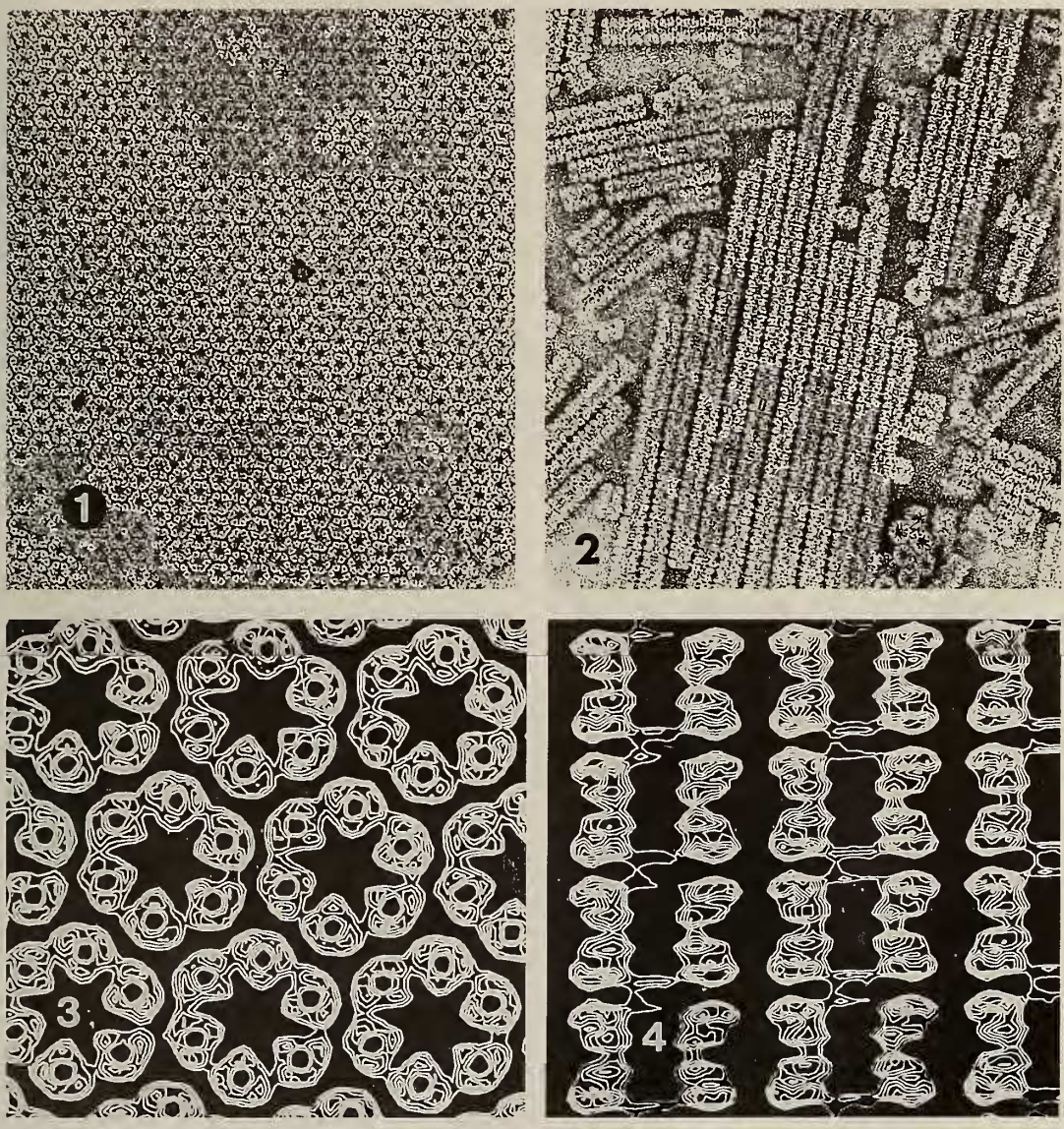


FIG. 2. Electron microscopic images of *Spinographis* chlorocruorin. 1 and 2 are bidimensional crystals in the axial and lateral projections, respectively. 3 and 4 are the relative computed reconstructions for the axial and lateral projections, respectively. Magnification electron micrographs 90,000×. Note the tetrahedral structure of each submultiple. (By courtesy of Dr. Ghiretti Magaldi, A. [22]. Reprinted with permission from Springer Verlag.)

of biological macromolecules in solution. For instance, the radius of gyration and maximum dimension of *Tylorrhynchus* hemoglobin have been determined to be 10.8 ± 0.2 nm and 29.6 ± 0.5 nm, respectively, as listed in Table 2 in comparison with values for *Lumbricus* hemoglobin [70]. These molecular parameters are in good agreement with the values obtained from the STEM images. A model that fits the X-ray scattering curve of *Tylorrhynchus* hemoglobin is shown in Figure 3 [70]. This model indicates some protein masses in the center of the molecule as well as betweem submultiples consistent with STEM images. However, it should be noted that in this model the small

TABLE 2. Radii of gyration and maximum dimensions of *Tylorrhynchus* and *Lumbricus* hemoglobins determined by small-angle X-ray scattering

Hemoglobin	Radius of gyration (nm)	Maximum dimension (nm)	Ref.
Tylorrhynchus	10.8±0.2	29.5±0.5	[70]
Lumbricus	11.2±0.2	29.0±1.0	[67]

(from Pilz, I., Schwarz, E., Suzuki, T. and Gotoh, T. [70]. Reprinted with permission of the publishers, Butterworth & Co. Ltd. ©.)

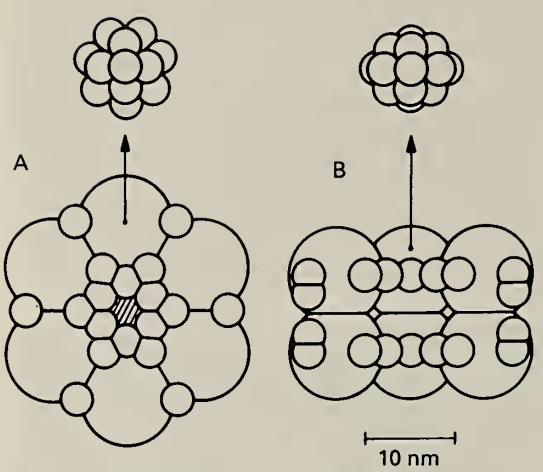


FIG. 3. A model of *Tylorrhynchus* hemoglobin based on the X-ray scattering curve. A and B are top and side views, respectively. Each submultiple indicated by a large sphere is composed of 17 small spheres arranged in five tiers containing one, four, seven, four and one spheres, respectively. There are also four small spheres per submultiple located between the large spheres. Thus the whole model contains 12 ×21=252 small spheres. (from Pilz, I., Schwarz, E., Suzuki, T. and Gotoh, T. [70]. Reprinted with permission of the publishers, Butterworth & Co. Ltd. ©.)

spheres used to simulate the quarternary structure have no relation to the real number and size of the subunits or polypeptide chains forming the molecule. Furthermore, the model in which each submultiple consists of four tetramers of ellipsoids in a tetrahedral arrangement also fits all the SAXS data fairly well [70]. According to the SAXS analysis, the protein mass in the central hole of *Tylorrhynchus* hemoglobinis less than that *Lumbricus* hemoglobin [70]. In sharp contrast, measurement of the pixel intensity of STEM images indicates more protein mass in the central hole of *Tylorrhynchus* hemoglobin than in that of *Lumbricus* hemoglobin [11]. Thus, the technical limitations of STEM, CTEM and SAXS must be taken into account in considering the filamentous structure in the central cavity.

MOLECULAR PHYLOGENY

The hemoglobin of the polychaete Tylorrhynchus was the first multi-subunit extracellular hemoglobin to be sequenced completely [9]. Soon afterwards, the corresponding chains of the oligochaete *Lumbricus* hemoglobin were sequenced [13, 14]. These sequencings provided much information on the phylogeny of giant annelid hemoglobins. Two chains from other multisubunit oligochaete hemoglobins have subsequently been sequenced [71, 72]. Figure 4 shows the amino acid sequences of the four chains of *Tylorrhynchus* hemoglobin [5–7, 9]. Nine of the 25 invariant residues are also conserved in the human β chain. In vertebrate hemoglobins [73, 74], all these residues except Val (A8) and Trp (A12) belong to the central exonic regions, which are known to be the minimal functional entity for O_2 binding [75]. It is noteworthy that the invariants of *Tylorrhynchus* hemoglobin involve the residues corresponding to the distal (E7) His, distal (E11) Val, and proximal (F8) His of vertebrate hemoglobins, which are the most important residues for maintaining the functional properties. The fifth coordination position of the iron atom in the heme is known to be histidine F8 (the proximal His); O_2 is bound at the sixth coordination position. In sperm whale myoglobin, the distal (E7) His is known to be the only residue capable of interacting directly with bound dioxygen and stabilizing it [76]. All eight chains of *Tylorrhynchus* and *Lum-*

bricus hemoglobins are clearly homologous with those of vertebrate hemoglobins [9, 13, 14]. In fact, each of the isolated chains shows a typical absorption spectrum of a globin chain [46, 77, 62]. Jhiang *et al.* [78] found that the gene of a heme-containing chain of *Lumbricus* hemoglobin has a two intron-three exon structure like those of vertebrate globin chains [73]. Therefore, there is no reason to maintain the old name 'erythrocruorin' for invertebrate hemoglobins except familiarity with this name, as pointed out by Garlick and Riggs [12]. The heme moiety of 'erythrocruorin' is the same as that of a vertebrate hemoglobin [79].

As the giant annelid hemoglobins differ from other hemoglobins in possessing disulfide-bonded 'trimers' or 'tetramers', the sites of half-cystine residues in *Tylorrhynchus* and *Lumbricus* hemoglobins are noted to be all located in the side exonic regions [8, 9, 11, 14, 78], as shown in Figure 4. These residues all participate in forming either intra- or inter-chain disulfide bridges [11, 14], as shown in Figure 5. There is one intra-chain disulfide bridge in each chain of *Lumbricus* and *Tylorrhynchus* hemoglobins including the monomers [11, 14]. As the multi-subunit annelid hemoglobins dissociate completely into separate chains in the presence of a reducing agent without any other protein-denaturant [21, 81, 82], the disulfide bonds appear to have a key role in the *in vivo* assembly of the giant molecule [11]. Namely, point mutations at the positions now having Cys residues must have been a major factor in bringing about formation of these huge proteins. The side exons can also be considered to have evolved with a special role of stabilizing the molecular assembly.

High homologies are seen between *Tylorrhynchus* chains *b* and *B* (74 identical residues, 50% homology), and *Tylorrhynchus* chains *a* and *A* (57

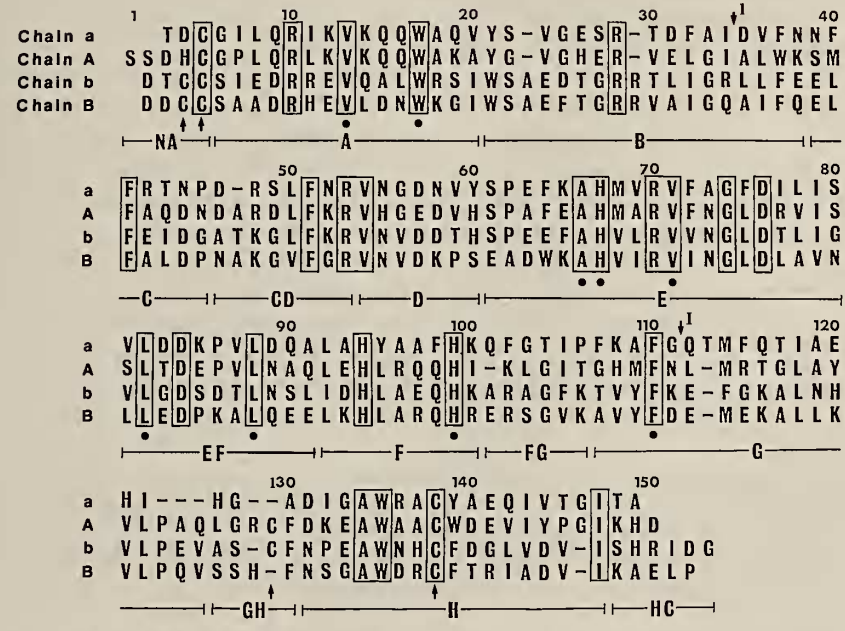


Fig. 4. Amino acid sequences of the constituent chains of *Tylorrhynchus* hemoglobin. The alignment is based on the assumption that the helical segments present in most other globins are also present in the constituent chains of the multi-subunit annelid hemoglobin. The boxed residues indicate the 25 invariable residues in four globin chains. The residues indicated by a *dot* are homologous with those of the human β chain. Arrows without a suffix indicate the positions of half-cystine residues. Arrows with the suffix '*I*' indicate intronic positions assuming that the intronic positions in *Tylorrhynchus* hemoglobin are the same as those in vertebrate globins [73] and *Lumbricus* hemoglobin [78]. (from Suzuki, T. and Gotoh, T. [9]. Reprinted with permission from the American Society for Biochemistry and Molecular Biology.)

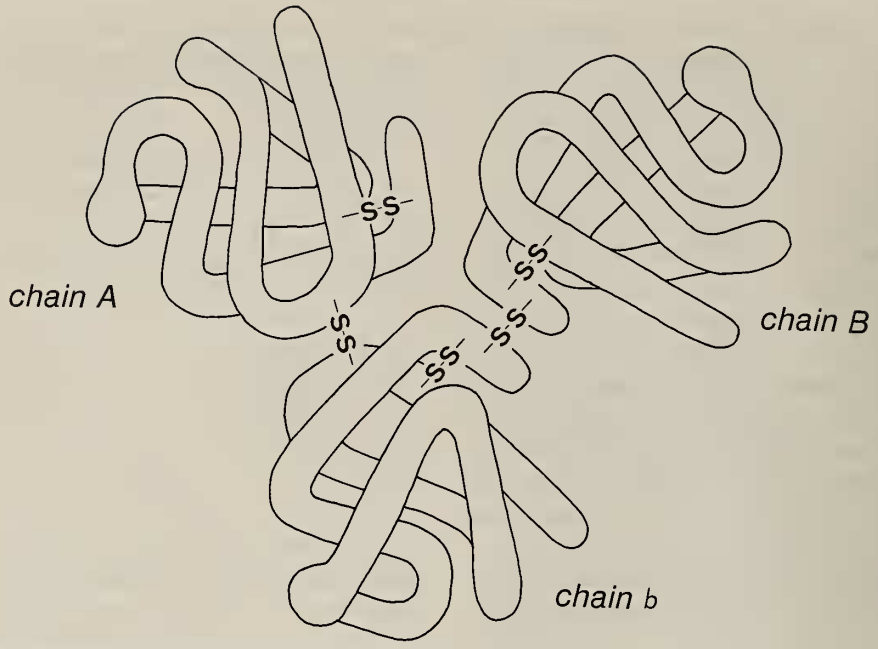


FIG. 5. A model for the steric assembly of the disulfide-bonded 'trimer' of chains *A*, *b* and *B* in *Tylorrhynchus* hemoglobin. 'Globin folding' was cited from that of the human β chain [80]. (from Suzuki, T., Kapp, O. H. and Gotoh, T. [11]. Reprinted with permission from the American Society for Biochemisrty and Molecular Biology.)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Lum. a						Glu	CYS	Leu	Val	Thr	Glu	Gly	Leu	Lys	VAL	Lys	Leu	Gln	TRP	Ala	Ser	Ala
Lum. A				Lys	Lys	Gln	CYS	Gly	Val	Leu	Glu	Gly	Leu	Lys	VAL	Lys	Ser	Glu	TRP	Gly	Arg	Ala
Tyl. a					Thr	Asp	CYS	Gly	Ile	Leu	Gln	Arg	Ile	Lys	VAL	Lys	Gln	Gln	TRP	Ala	Gln	Val
Tyl. A			Ser	Ser	Asp	His	CYS	Gly	Pro	Leu	Gln	Arg	Leu	Lys	VAL	Lys	Gln	Gln	TRP	Ala	Lys	Ala
Lum. b	Asp	Glu	His	Glu	His	Cys	CYS	Ser	Glu	Glu	Asp	His	Tyr	Ile	VAL	Gln	Lys	Gln	TRP	Asp	Ile	Leu
Lum. B	Ala	Asp	Glu	Glu	Ser	Cys	CYS	Ser	Tyr	Glu	Asp	Arg	Arg	Glu	VAL	Arg	His	Ile	TRP	Asp	Asp	Val
Tyl. b				Asp	Thr	Cys	CYS	Ser	Ile	Glu	Asp	Arg	Arg	Glu	VAL	Gln	Ala	Leu	TRP	Arg	Ser	Ile
Tyl. B				Asp	Asp	Cys	CYS	Ser	Ala	Ala	Asp	Arg	His	Glu	VAL	Leu	Asp	Asn	TRP	Lys	Gly	Ile

NA — A

FIG. 6. Alignment of NH_2-terminal sequences of *Tylorrhynchus* and *Lumbricus* chains. The boxed residues indicate invariant residues in the eight chains and either the upper strain (strain *A*) or the lower strain (strain *B*). The residues indicated by a *dot* are homologous with those of the human β chain. Others are as for in Fig. 4. (from Gotoh, T., Shishikura, F., Snow, J. W. Ereifej, K. I., Vinogradov, S. N. and Walz, D. A. [24]. Reprinted with permission from the Biochemical Society and Portland Scientific Press.)

identical residues, 40% homology). Therefore, phylogenetically, the 'trimeric' chain *A* is more closely related to the 'monomeric' chain *a* than to either of the other 'trimeric' chain, *b* and *B* [9]. This finding has been extended to the idea that there are in general two distinct groups, strain *A* and *B*, of chains in multi-subunit annelid hemoglobins, as clearly seen in Figure 6 [24]. The separation of these strains must have been derived from the gene duplication [24]. In fact, using the unweighted pair-group clustering method [83], Fushitani *et al.* [14] clearly demonstrated two

TABLE 3. Proposed common nomenclature for the chains of multi-subunit annelid hemoglobins, and the corresponding names for *Tylorrhynchus* and *Lumbricus* hemoglobins used hitherto

Hemoglobin		Chain	name		Ref.
Tylorrhynchus	I	IIA	IIC	IIB	[6]
Lumbricus	I	II	III	IV	[55]
Lumbricus	*d*	*b*	*c*	*a*	[77]
Common name	*a*	*A*	*b*	*B*	This paper
No. of Cys	2	3	4	3	[9, 14]

The arbitrary names were given according to either the order of mobility on SDS-PAGE [6, 55] or the elution order on column chromatography [6, 77]. The proposed nomenclature is based on the homology between different hemoglobins.

subfamilies in the phylogenetic tree for *Lumbricus* and *Tylorrhynchus* hemoglobins. Furthermore, they used the type of inter-chain disulfide bonding and the number of half-cystine residues to distinguish two types of chains in the same strain [14]. Thus the correspondence of the four constituent chains in these two hemoglobins has been established.

Here, we would like to propose a new nomenclature for common names of the sequenced chains to facilitate comparison of the 'monomeric' and 'trimeric' globin chains of different species. We have used the new nomenclature without definition in Figures 4, 5 and 6. The smaller and 'monomeric' chain in strain *A* is named chain '*a*', and the other one in strain *A* is named chain '*A*'. Chain *a* (139 residues) of *Tylorrhynchus* hemoglobin is smaller than all the other constituent chains: chain *A*, 146 residues; *b*, 149 residues; *B* 148 residues. *Lumbricus* chain *a* is also the smallest constituent and exists as a 'monomer' [13, 14, 55]. Chains *a* and *A* can also be distinguoshed by the number of half-cystine residues [9, 14]: they have 2 and 3 half-cystine residues, respectively, as seen in Figure 4. Similarly, chains '*b*' and '*B*' in strain *B* can be defined as those having 4 and 3 half-cystine residues, respectively. Chains *b* was shown experimentally to be situated in the center of the trimer '*AbB*', as shown in Figure 5 [11, 14]. Employing the myoglobin-fold for each chain in the steric model of disulfide-bonded 'trimer', five disulfide bridges can easily be located without any bending or stretching [11]. Chain *a* exists as a 'monomer' because it has only two half-cystine residues and forms one intra-chain disulfide bond [9, 11, 14]. The distributions of half-cystine residues in *Lumbricus* and *Tylorrhynchus* hemoglobins are the same [9, 14]. The relationships between the new names and the arbitrary ones used previously for the *Tylorrhynchus* and *Lumbricus* chains are summarized in Table 3. As the polychaete *Tylorrhynchus heterochaetus* and the oligochaete *Lumbricus terrestris* are very different species each other in the phylum Annelida, the proposed nomenclature can be extended to many other annelid hemoglobins. A slight modification of the proposed nomenclature will, however, be necessary in the future bacause some annelid hemoglobins and chlororuorins contain disulfide-bonded 'tetramers' [30].

Figure 7 shows the phylogenetic tree of the nine globin chains of the multi-subunit annelid hemoglobins from the polychaete *Tylorrhynchus* and the oligochaete *Lumbricus* and *Pheretima sieboldi* according to the unweighted pair-group clustering method. Strains *A* and *B* are clearly separated. According to the unweighted pair-group clustering method [83], the amino acid substitution rate for the 'functionally essential' central exonic region is about 1.5 times slower than that for the 'structurally essential' side exonic regions [71]. The observed identities of the nine chains of *Tylorrhynchus, Lumbricus* and *Pheretima* hemoglobins range from 32 to 51%, as shown in Figure 7. Some of these identities are comparable to that (44%) between the α and β chains of human hemoglobin, which were separated by gene duplication about 450 million years ago in the Ordovician period [84].

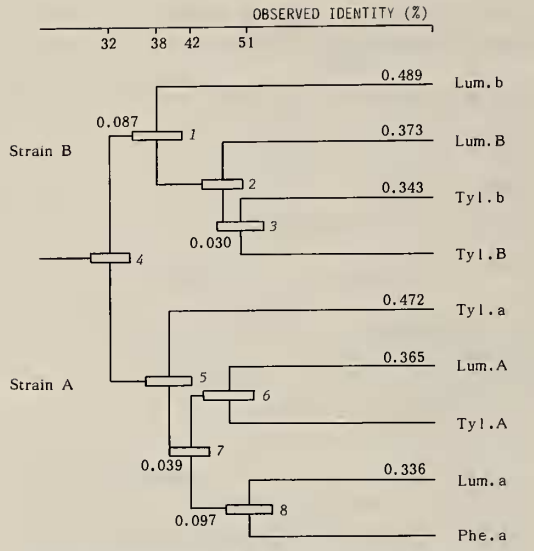


FIG. 7. Phylogenetic tree of the nine heme-containing chains from multi-subunit annelid hemoglobins. The tree was constructed from a homology matrix by comparison of 137 amino acid residues common to all chains [71], by an unweighted pair-group clustering method [83]. The standard errors at the branching points 1–8 are 0.045, 0.038, 0.042, 0.035, 0.041, 0.044, 0.036 and 0.042, respectively.

Assuming comparable evolutionary rates for extracellular and vertebrate hemoglobins, Fushitani *et al.* [14] suggested that the divergence of the Oligochaeta and Polychaeta occurred around the time of the gene duplication that led to the α and β gene families in vertebrates. On the other hand, from the maximum parsimony tree, Goodman *et al.* [28] calculated the times when the annelid globin chains were separated more exactly. For instance, they reported that the *Tylorrhynchus* chain *b* and *B* were separated about 140 miliilon years ago in the Cretaceous period, the *Tylorrhynchus* chain *a* and other *Tylorrhynchus* chain were seperated about 380 million years ago in the Devonian period, and the *Glycera* intracellular monomeric chain and extracellular globin chains were separated about 575 million years ago in the Cambrian period. Although it is of great biological interest to know the time when the Polychaeta and Oligochaeta were separated, fossil records of annelids, and particularly of oligochaetes are very incomplete [85].

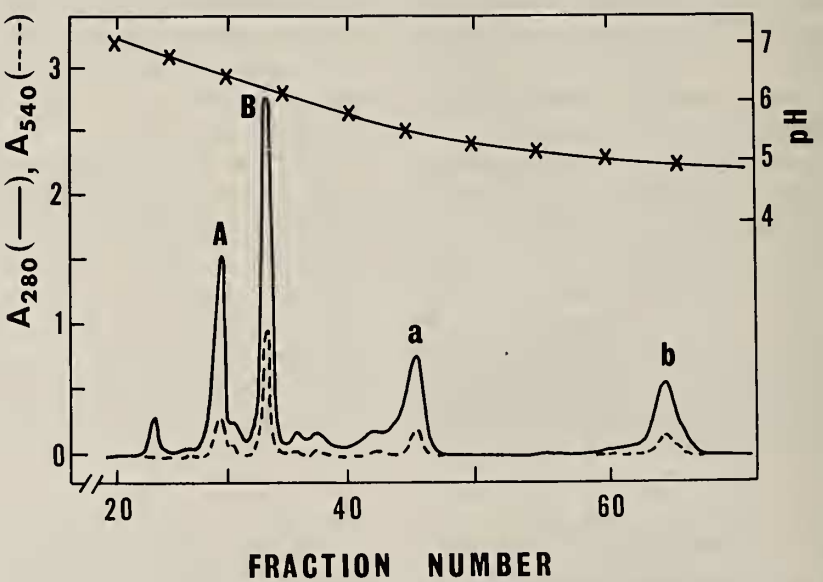
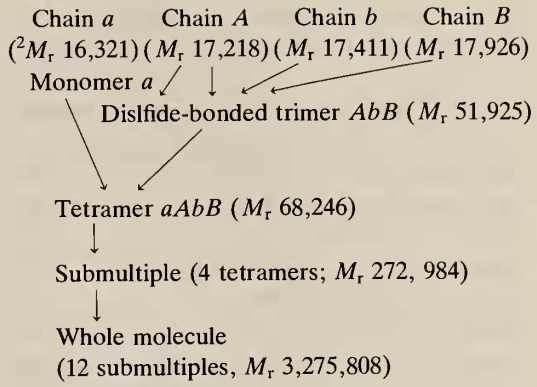


FIG. 8. Elution profile on chromatofocussing of *Tylorrhynchus* cyanomethemoglobin. Hemoglobin solution was reduced with dithiothreitol and applied to a PBE94 column equilibrated with a imidazole buffer (pH 7.4). Material was eluted with Polybuffer 74 (pH 5). Peaks A, B, a and b are those of chains *A*, *B*, *a* and *b*, respectively. Note that all four chains isolated carry a heme. (from Suzuki, T., and Gotoh, T. [6]. Reprinted with permission from the American Society for Biochemistry and Molecular Biology.)

'192-CHAIN' MODEL OF THE SUBUNIT ASSEMBLY

Sequence analyses of the multi-subunit annelid extracellular hemoglobins have indicated the precise molecular weight of protein moiety in each constituent chain, which is essential for construction of a model of the molecular architecture. The heme content of each chain has been clarified directly by column chromatographic isolation procedures [6, 24, 77, 86]. Figure 8 shows the elution profile of each constituent chain of *Tylorrhynchus* hemoglobin on chromatofocussing [6]. The isolated chains exhibit the typical absorption spectrum of a vertebrate myoglobin. Therefore, it is concluded that all four chains, *a*, *A*, *b* and *B*, contain one heme group per chain. In fact, these four chains have a histidine residue, which corresponds to the proximal one (F8) of vertebrate hemoglobins [26], as shown in Figure 4.

The molar ratio of the four *Tylorrhynchus* chains *a*:*A*:*b*:*B* was determined to be nearly 1:1:1:1 by statistical comparison of the exact amino acid compositions calculated from the sequence of each chain and the observed composition measured by amino acid analysis of the whole molecule [10]. Considering the apparent molecular masses of the whole molecule (3, 370 kDa), submultiple (250 kDa), unstable tetramer (72 kDa) and disufide-bonded trimer (50 kDa) [9, 87, 88], we proposed that the formation of *Tylorrhynchus* hemoglobin may be as follows [10].



Therefore, our model proposed for *Tylorrhynchus* hemoglobin consists of 192 polypeptide chains containing heme. According to the new nomenclature proposed above, *Tylorrhynchus* hemoglobin can be represented as '$(aAbB)_{48}$'. *aAbB* is the minimum structural entity, which may correspond to one unit in the tetrahedral structure of the submultiple observed in the STEM image (Fig. 1). This giant protein consists of 27,936 amino acid residues and 192 heme groups, and is linked by 288 disulfide bridges, 192 intra- and 96 inter-chain disulfide bonds. These are the contents of our symmetrical '192-chain' model. The salient point of this model is that the subunit assembly is based on the exact molecular weight and strict molar ratio of each constituent polypeptide chain. The '192-chain' model is in good agreement with electron microscopic observations for the tetrahedral structure of a submultiple [21, 22, 61, 64, 89].

TABLE 4. Earlier models of annelid multi-subunit hemoglobins

Hemoglobin	Minimum M_r per heme	No. of units	M_r of whole molecule ($\times 10^{-6}$)	Ref.
Lumbricus	17,250	144	2.48	[1]
Lumbricus	17,600	192	3.38	[3]
Arenicola	27,000	192	2.59	[54]
Octolasium	23,000	144	3.30	[57]
Lumbricus	17,000	192	3.26	[90]
earthworm	22,000	192	3.84	[58]
Tylorrhynchus	17,062	192	3.275808	[10]

All models except those reported in refs. [10] and [54] consist of a single species of unit protein containing a heme moiety.

[2] The M_r of each constituent chain of *Tylorrhynchus* hemoglobin including a heme group was recalculated.

However, this model does not explain the filamentous structure in the central hole of the molecule, shown in Figure 1. Two further other problems about the '192-chain' model are that the measured value of the minimum molecular mass per heme is much higher than the value calculated from the model, and that the SDS-PAGE pattern often shows one or two extra minor components with molecular masses of about double those of the chains containing heme [88].

Table 4 shows some earlier models of annelid hemoglobins compared with our '192-chain' model. Chiancone *et al.* [57] and David and Daniel [58] reported higher values for the minimum molecular weight. These values might be reasonable for the minimum molecular weight per heme, but not for the molecular weight of a minimum unit, because sequence analyses of *Tylorrhynchus* [9] and *Lumbricus* hemoglobins [14] revealed that the size of each chain containing heme is comparable with that of myoglobin. Chiancone *et al.* considered the '144-chain' model based on the relationships of the molecular weights of the minimum unit and whole molecule. On the other hand, David and Daniel proposed a '192-chain' model by which the calculated molecular weight of the whole molecule reached the measured value of 3.84×10^6, in contrast to the others clustered in the first half of 3 million. Anyway, it is now obvious that these models overestimated the molecular weight of a minimum unit. On the other hand, in most cases, the molecular mass of a constituent chain was underestimated by SDS-PAGE. Waxman [54] examined *Arenicola cristata* hemoglobin by SDS-PAGE and he found two major components with molecular masses of 13 kDa and 14 kDa. As the minimum molecular mass per heme was estimated to be 27 kDa, he proposed a '192-chain' model for *Arenicola* hemiglobin with a relatively lower molecular mass of 2,590 kDa, which consists of two species of constituent chains, half of which do not contain a heme group. Vinogradov and his colleagues have maintained the idea that not all chains contain a heme group [16, 55, 91].

'BRACELET' MODEL OF THE SUBUNIT ASSEMBLY

Shlom and Vinogradov [55] noted fifth and sixth components of multi-subunit hemoglobins that can always be observed as faint bands V and VI on SDS-PAGE of a preparation of *Lumbricus* hemoglobin. As these components are about twice the size of the major components, chains *a*, *A*, *b* and *B*, they are designated as 'D1' (M_r=31,000) and 'D2' (M_r=37,000) and called "dimers" arbitrarily (15). Recently, Vinogradov's group [15–17] proposed a 'bracelet' model for the subunit assembly of a giant annelid hemoglobin in which D1 and D2 have the remarkable role of linking 12 submultiples. In the novel 'bracelet' model, subunits D1 and D2 are assumed to form a closed circular collar or bracelet decorated with 12 complexes of several 'monomers' *a* and 'trimers' *AbB*, providing the electron microscopic appearance of a symmetrical hexagonal bilayer. There is no inter-chain disulfide bridge between 'dimers' D1 and D2, and these and heme-binding subunits *a* and *AbB* [55]. The 'bracelet' model was deduced from extensive studies on the dissociation and reassociation of *Lumbricus* hemoglobin either at an extreme of pH [17, 19] or in the presence of a dissociating agent at neutral pH [16]. The existence of subunits D1 and D2 appeared to be essential for complete reassociation of the molecule of *Lumbricus* hemoglobin from the dissociated products under physiological conditions. Although the experimental results did not provide direct proof of the existence of a bracelet structure, the filamentous structure in the central hole of the giant hemoglobin may corres-

Table 5. Models of annelid hemoglobins with none-heme chains

Hemoglobin	No. of chain	No. of heme	M_r ($\times10^{-6}$)	Ref.
Arenicola	192	96	2.59	[54]
Lumbricus	204	144	3.77	[91]
Lumbricus	*ca.* 200	156	3.8	[16, 20]
Lumbricus	204	192	3.77	[23]

pond to the bracelet [20]. An alternative explanation is that subunits D1 and D2 do not form a continuous bracelet structure but may act as linkers between submultiples [16].

Table 5 summarizes the models of multi-subunit annelid hemoglobins that include non-heme chains. These models depend greatly upon the estimated value for the heme content, which varied significantly in different laboratories. Vinogradov *et al.* [15, 91] assumed that one chain of the 'trimer' of *Lumbricus* hemoglobin did not contain heme. However, this idea was disproved by the finding that all chains isolated by chromatography under mild conditions contained a heme group [24, 77]. Recently, Fushitani *et al.* [23] determined the heme content of *Lumbricus* hemoglobin to be one mole per 19,000 g of protein. Their model consists of 48 '*aAbB*' and 12 chains of D1 and D2, as shown in Table 5. This model appears to be the most consistent with all data so far obtained on *Lumbricus* hemoglobin. Fushitani and Riggs [23] assumed that D1 and D2 contain no heme, and reported that the amounts of D1 and D2 constitute 11.2% of the total. On the other hand, Mainwaring *et al.* [17] reported that D2 exhibits a reduced capacity to bind heme. The relative proportions of D1 and D2 in *Lumbricus* hemoglobin are reported to be 25 and 10%, respectively [17, 55]. D1 was separated into two fractions [92]. There are no homologies in the reported NH_2-terminal sequences of D2 and two D1 chains or between these and those of other globins [23, 92]. If D1 and D2 have evolved as heterogeneous components with the same function of linking the complexes of subunits *a* and *AbB* together, they should exhibit high homology in terms of amino acid sequences. The natures of chains D1 and D2 are still unclear. Comparative studies revealed diversity in the appearance of D1 and D2 components [30]. In *Nephthys incisa* hemoglobin, no dimer component can be seen in the absence of a reducing agent, but two dimers are seen on SDS-PAGE in the presence of a reducing agent [93]. On the other hand, in the case of *Potamia leptochaeta* chlorocruorin, one band of dimer is seen in the absence of a reducing agent, but no dimer is seen in reduced conditions [94]. Waxman [54, 61] suggested that the faint bands of D1 and D2 might be those of partially reduced dimers derived from disulfide-bonded 'tetramers'. Pionetti and Pouyet [95] throught that the two bands with molecular masses of about 30 kDa must be due to contaminants with some non-heme chains. At present, it can not be concluded that D1 and D2 are common to all multi-subunit annelid hemoglobins, although several lines of evidence support the 'bracelet' model.

According to the '192-chain' model and the 'bracelet' model, the values for the calculated molecular weight of *Lumbricus* hemoglobin are 3,343,872 and 3,770,000, respectively [23]. Ghiretti Magaldi *et al.* [21] stated, "we have absolutely no reason to believe that these M_r values are underestimated by even 25%". They determined the relative molecular weights of *Opheria bicornis* hemoglobin and *Spirographis sparanzanii* chlorocruorin to be about 3×10^6. In contrast, Vinogradov and Kolodziej [53] stressed that the recent values reported for the molecular weight of *Lumbricus* hemoglobin are clustered at about 3.8×10^6. Furthermore, Vinogradov *et al.* [16] stated in the 'bracelet' model, "it is postulated that the stoichiometries of some of the subunits need not be constant". Due to the symmetrical appearance of 12 submultiples, however, we believe that there are at least 6 or 12 units for each constituent in the whole molecule. From an X-ray crystallographic study, Royer and Hendrickson [96] reported that self-rotation function calculations of *Lumbricus* hemoglobin reveal D_6 symmetry to a resolution of at least 6 Å.

PERSPECTIVE

Which is correct, the '192-chain' model or the 'bracelrt' model? At present neither can be disregarded. The discrepancy between these models will be explained in part by the results of sequencing the D1 and D2 chains from at least two species of hemoglobins. Immuno-electron microscopy might be a suitable method to determine the loci of D1 and D2 in the molecule, if it is possible to distinguish D1 and D2 from the other chains *a*, *A*, *b* and *B* immunologically [97]. The fine three-dimensional structure of the multi-subunit hemoglobin can of course be determined by X-ray

crystallography, which is currently in progress using crystals of *Lumbricus* hemoglobin [96, 98]. Comparative study will also be of great importance in considering D1 and D2. In fact, it is noteworthy that the deep-sea tube worm *Lamellibrachia sp.* contains two kinds of hemoglobins with molecular masses of 440 kDa and 3,000 kDa, the latter having the characteristic quarternary structure of multi-subunit annelid hemoglobins [99, 100]. Suzuki *et al.* [101] found that the larger one contains components corresponding in molecular size to 'linkers' D1 and D2, whereas the smaller one does not contain 'linker' proteins. Although the tube worm has been assigned to the phylum Vestimentifera [102], the NH_2-terminal amino acid sequences of its four chains containing heme exhibit high homology with those of polychaete and oligochaete chains *a*, *A*, *b* and *B* [101, 103]. Even more important, the 'linker' chain D1 of the tube worm hemoglobin shows slight, but significant homology with at least one of the four heme-binding chains sequenced completely [103, 104]. The alignment of the D1 chain with the heme-binding chain suggests that the linker resulted from gene duplication and exon shuffling of a globin chain with a three exon-two intron structure. The D1 chain consists of two domains and the first exon of domain 1 and the last exon of domain 2 are deleted. Therefore, there is a high possibility that in *Lumbricus* hemoglobin either D1 and D2 is also a real member of the molecule. Anyway, to find a rare protein, it is essential to scrutinize any possible candidates thoroughly. Conversely, when morphologists agree with the idea of biochemists that tube worms belong to the phylum Annelida, the sequences of the D1 chain of *Lamellibrachia* hemoglobin [104] will be recognized as the first 'dimer' chain of annelid hemoglobins sequenced.

In order to understand much clearly about the evolution of multi-subunit annelid hemoglobins, it is necessary to investigate the locus of each gene of the constituent chains in the DNA molecules (S). There are many challenging problems on invertebrate hemoglobin molecules to be solved in terms of DNA sequences. In the near future, the drastic mutations occurred in soluble proteins during the course of evolution can be explained in large parts by the knowledge of invertebrate hemoglobins because they contain treasures of diversity not only in the primary and quarternary structures [30, 32, 105–107] but also in the biosyntheses including the regulation [108, 109]. On the other hand, it will also be of great interest to design disulfide-bonded trimers and tetramers such as '$\alpha\ \beta\ \alpha$', '$\beta\ \alpha\ \beta$', and '$\alpha_2\ \beta_2$' of vertebrate hemoglobins, which might be synthesized with the technique of DNA recombinant [110] by inserting half-cystine residues at sites corresponding to those of Cys-4, 5, 129 and 148 in multi-subunit annelid hemoglobins.

ACKNOWLEDGMENTS

This paper is dedicated to Professor Kazuhiko Konishi of the Department of Biology, Faculty of Science, Tohoku University on the occasion of his retirement. He has encouraged us throughout this work.

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